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Biochimica et Biophysica Acta 1359 (1997) 222–232

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Sulindac sulfide induces several subpopulations of colon cancer cells, defined by PCNA/Ki-67 and DNA strand breaks

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Received 5 May 1997; accepted 14 July 1997

Abstract

We assessed the effect of sulindac sulfide (SS), a colon cancer chemopreventive agent, on the proliferation and apoptosis in the colon cancer cell lines HCT-15 and HT-29. We applied a triparameter flow cytometric analysis that simultaneously determined DNA content, expression of Ki-67 or proliferating cell nuclear antigen (PCNA), and extent of DNA strand breaks by TUNEL (TdT-mediated dUTP nick end labeling). HCT-15 and HT-29 cells were exposed to SS 200 μM and 175 μM, respectively, for up to 72 h. As expected, SS inhibited proliferation and induced apoptosis. SS also induced several subpopulations of cells defined by their expression of proliferation markers and DNA strand breaks. By 72 h the rapidly proliferating cells [PCNA/Ki-67(+)/TUNEL(−)] were reduced from > 90% to about one third. Of the remaining cells, about one third were apoptotic [PCNA/Ki-67(−)/TUNEL(+)] and one third were quiescent [PCNA/Ki-67(−)/TUNEL(−)]. Another subpopulation was detected that was PCNA/Ki-67(+)/TUNEL(+), some had a dominant subdiploid peak and over half were in S or G_2/M phases by DNA content. Thus, a subpopulation of apoptotic cells strongly expressed PCNA and Ki-67, suggesting that their specificity as proliferation markers may need reassessment. Similar results were obtained with the HL-60 promyelocytic cell line. © 1997 Elsevier Science B.V.

Keywords: Sulindac sulfide; Apoptosis; Colon cancer; Proliferation; Biomarker; Cell cycle

1. Introduction

One of the most remarkable advances in the prevention of colon cancer is the appreciation of the prophylactic effect of nonsteroidal antiinflammatory drugs (NSAIDs). Animal, clinical and epidemiological observations have documented the ability of these compounds to not only reduce the incidence of and mortality from colon cancer, but also, in some cases, to regress already formed tumors. It is, therefore, understandable that an intense effort is underway to determine the mechanisms by which they bring about this effect.

Sulindac, and its metabolites, such as sulindac sulfide (SS), produce a wide range of effects on cells that could be useful in colon cancer prevention. Their

Abbreviations: SS, sulindac sulfide; NSAIDs, nonsteroidal antiinflammatory drugs; PCNA, proliferating cell nuclear antigen; TUNEL, TdT-mediated dUTP nick end labeling; 7AAD, 7-amino-actinomycin D; TdT, terminal deoxynucleotidyl transferase; BSA, bovine serum albumin; PBS, phosphate buffered saline

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PII S0167-4889(97)00102-X

mechanism of action has been studied by our laboratory and others. It is now well documented that these compounds exert two powerful effects on cultured colon cancer cells; they: (1) inhibit cell proliferation, and (2) induce cell death by apoptosis [1,2]. The apoptotic effect has also been documented in the mucosa of patients with familial adenomatous polyposis treated with oral sulindac [3].

Both proliferation and apoptosis are critical determinants of the growth of a tumor. Indeed, the growth rate of tumors is determined by the proliferation rate and by the rate of death of the tumor cells. Besides its significance for tumor biology, the proliferation rate is an important prognostic indicator for various cancers, including colon cancer tissues [4].

The proliferation rate of tumors has been monitored by using various proliferation markers. Prominent among the proliferation markers are the Ki-67 antigen [5–9] and proliferating cell nuclear antigen (PCNA) [10–15]. The Ki-67 antigen, which is detected in all phases of the cell cycle except in G_0 [7], proved excellent for the estimation of the growth fraction in a wide variety of normal and malignant human tissues, including colon [16]. The MIB-1 antibody, frequently used in such studies, was generated against recombinant parts of the Ki-67 antigen [17]. PCNA, an auxiliary protein to DNA polymerase ∂ [18], functions as a co-factor in DNA synthesis. The synthesis and expression of PCNA are enhanced in proliferating cells [19]. PCNA has been considered as the most reliable method to evaluate proliferation in colon tissues [4].

Apoptotic cell death, a process of gene directed self-destruction [20,21], can affect the cell number in a tissue. Apoptosis involves activation of endonucleases which causes DNA strand breaks and, ultimately, DNA fragmentation in situ [22]. In this context as well, DNA damage and repair are of fundamental importance for the continued survival of the cell [23].

As we were studying the mechanism of the apoptotic and anti-proliferative effects of SS, we obtained data suggesting that Ki-67 and PCNA may be expressed in apoptotic (dying) cells. This discovery could have wider implications for (a) the mechanism of action of SS, and (b) the utility of these antigens as proliferation markers. Because of these considerations, we undertook a detailed study of this preliminary observation. The results of our work are presented in this paper.

2. Materials and methods

2.1. Cell lines

The human colon adenocarcinoma cell lines HT-29 and HCT-15 cells and the promyelocytic leukemia cell line HL-60 were obtained from American Type Culture Collection (ATCC, Rockville, MD), and grown according to the instructions of ATCC. HCT-15 cells and HL-60 cells were cultured in RPMI 1640, and HT-29 cells in McCoy 5A (GIBCO, Grand Island, NY) media, which were supplemented with 10% fetal calf serum (Gemini Bio-Products, Calabasas, CA), streptomycin (10 000 U/ml) and penicillin (10 000 U/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded at a density of 1×10^6 cells per 100 cm^2 culture dish (Falcon, Becton-Dickinson, NJ).

2.2. Reagents

Sulindac sulfide (desoxy-sulindac, generously provided by Merck, Rahway, NJ), was dissolved in DMSO (Fisher Scientific, Fair Lawn, NJ). All compounds were added to the culture medium and cells prior to plating. The DMSO concentration was adjusted to 0.6% in all SS-supplemented media. The experiments were carried out with 2 independently prepared batches of SS. The batch used for HT-29 cells had optimal activity at 175 μM, whereas the batch used for the HCT-15 experiment had optimal activity in both cell lines at 200 μM.

2.3. Tricolor flow cytometric analysis

A tricolor flow cytometric staining technique was used in which DNA content was determined by 7-amino-actinomycin D (7AAD) (Sigma, St. Louis, MO) staining, Ki-67 and PCNA expression by phycoerythrin-conjugated mAbs, and DNA cleavage by the tailing reaction known as TUNEL (TdT-mediated dUTP nick end labeling), where fluorescein labels are incorporated in nucleotide polymers. Each of these procedures are described below.

2.4. Immunofluorescent staining for DNA strand breaks (TUNEL)

Cells were harvested by using 0.05% trypsin/EDTA (GIBCO) and then resuspended in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (PBS/BSA). Aliquots of cells were counted using a hemacytometer and tested for viability by the trypan-blue dye (Sigma) exclusion method. Cells that stained blue were considered ''non-viable''.

Cells (2×10^6) were washed with 1% PBS/BSA and fixed in 200 ml 2% paraformaldehyde (pH 7.4) in PBS for 15 min at 4°C. Cells were washed with PBS/BSA and incubated with 0.1% Triton-X 100 in PBS for 5 min on ice. A one-step procedure to detect DNA strand breaks was employed [24,25], using a commercial kit (Boehringer Mannheim, Indianapolis, IN); DNA strand breaks indicated apoptotic cells. In the procedure, the Enzyme Solution contained terminal deoxynucleotidyl transferase (TdT) and the Label Solution contained the labeled nucleotide in reaction buffer (3 nmol FITC-dUTP, 2 μl 25 mM $CoCl_2$ and TdT buffer (30 mM Tris pH 7.2, 140 mM sodium cacodylate). The cells were washed with PBS and resuspended in 50 μl/tube TUNEL Reaction Mixture (50 μl of Enzyme Solution plus 450 μl of Label Solution). Omission of TdT provided the negative control. Cells were incubated for 60 min at 37°C in a humidified atmosphere in the dark, washed with PBS/BSA and processed according to any of the following procedures.

2.5. Immunocytochemical staining for Ki-67 and PCNA

Following the above procedures, cells were incubated for 60 min at room temperature in the presence of primary antibodies: PCNA (PC-10) (diluted 1:200), (PharMingen, San Diego, CA); MIB-1 (anti-Ki-67), diluted 1:100, (Oncogene Sciences, Uniondale, NY); Nonspecific IgG_1 and $IgG_{2\alpha}$ which are the isotypic controls for PC-10 and MIB-1, respectively (Becton-Dickinson). Cells were then washed and incubated with goat-anti-mouse-phycoerythrin antibody (diluted 1:50, Jackson Immuno Research Lab, West Grove, PA) for 60 min at room temperature in the dark. Cell were washed again with PBS/BSA, resuspended in 10 μg/ml 7AAD in PBS and incubated at room temperature for 1 h prior to measurement.

2.6. Flow cytometry

Flow cytometry was performed using a Coulter Profile XL equipped with a single argon ion laser and FL1 (FITC); FL2 (phycoerythrin); FL3 (7AAD) filter combination. Minimal electronic compensation was used. The spectral properties of fluorescent probes were described previously [26]. A minimum of 10 000 events were collected for the particular subset analyzed for tricolor staining. All parameters (Linear FSC, Log SSC, Log of FITC, phycoerythrin and linear of 7AAD) were collected in listmode files. Data were further analyzed on an XL Elite Work station (Coulter).

3. Results

In this study we wished to assess the effect of sulindac sulfide on the status of DNA and the interrelationship of proliferation markers and apoptosis in colon cancer cells. To this end we first confirmed the (anticipated) effect of this compound on the kinetics of our cell lines, then described the expression of proliferation markers in relationship to DNA fragmentation (TUNEL positivity), and finally we assessed the effect of SS on these parameters.

3.1. Sulindac sulfide reduces the proliferation, alters the cell cycle distribution and induces apoptosis in HT-29 and HCT-15 colon cancer cells

2×10^6 HCT-15 or HT-29 colon cancer cells were exposed to sulindac sulfide at 200 μM and 175 μM, respectively, for up to 72 h. In agreement with our previous observations [1,2], SS dramatically reduced the proliferation of both cell lines. At 72 h, the number of HCT-15 cells was 10% of the control, and of HT-29 20% of the control. In both cell lines this effect was evident at 24 h and became maximal at 72 h.

Table 1 illustrates the effect of SS on cell cycle distribution of both cell lines. HCT-15 cells had no significant changes after treatment with SS. In contrast, the cell cycle distribution of HT-29 cells was

Table 1
The effect of sulindac sulfide on cell cycle distribution of HT-29 and HCT-15 colon cancer cells

Cell cycle phases	G_1	S	G_2/M
HT-29 cells			
Control	53%	35%	12%
Sulindac sulfide	87	8	5
HCT-15 cells			
Control	50	38	12
Sulindac sulfide	50	39	11

HCT-15 and HT-29 cells were treated with sulindac sulfide 200 μM and 175 μM, respectively, for 72 h. The percentage of cells in each phase of the cell cycle was determined by 7AAD staining, as described in Section 2. This protocol was followed three times and values each time were within 5%.

clearly altered by SS: the proportion of cells in G_1 phase was increased and that in S and G_2/M phases was decreased.

3.2. The presence of DNA strand breaks and the expression of proliferation markers define four populations of colon carcinoma cells

We employed a flow cytometric analysis of colon cancer cells that provided the simultaneous assessment of the following three parameters: (a) the expression of either Ki-67 or PCNA proliferation antigens; (b) the presence of DNA strand breaks (endonucleolysis), assessed by the TUNEL method, and (c) the distribution of the DNA content in the cell cycle, including the presence of the subdiploid (apoptotic) peak.

HCT-15 or HT-29 cells, plated in 100 cm^2 plates, were harvested 24, 48 and 72 h later and subjected to the triparametric analysis by flow cytometry. The type of results that were obtained are shown in Figs. 1 and 2; the relationship between DNA strand breaks

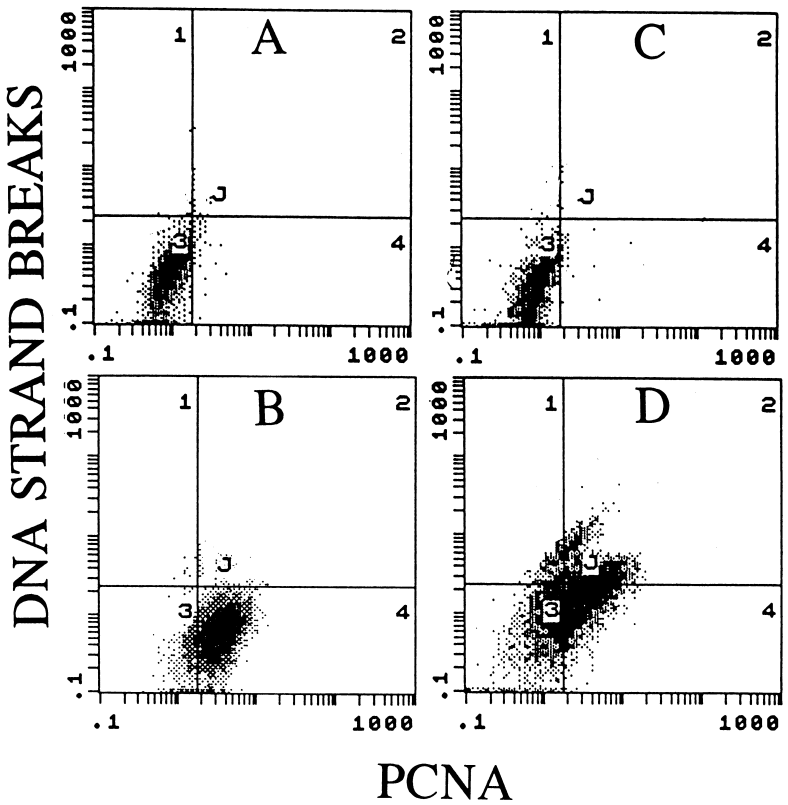

Fig. 1. DNA strand breaks and PCNA expression in HCT-15 colon carcinoma cells treated with sulindac sulfide. Cells were stained for PCNA and labeled with TUNEL, as in Section 2. Dot plots of TUNEL (ordinate) vs. PCNA (abscissa) are shown. Region 1, TUNEL(+)/PCNA(−); Region 2, TUNEL(+)/PCNA(+); Region 3, TUNEL(−)/PCNA(−); Region 4, TUNEL(−)/PCNA(+). Panel A: the untreated cells were stained with TUNEL control solution and $IgG_{2\alpha}$ (PCNA isotypic control). Panel B: untreated cells were stained with TUNEL and PCNA. Panel C: cells treated with SS 200 μM for 48 h were stained with TUNEL control solution and $IgG_{2\alpha}$. Panel D: cells treated with SS 200 μM for 48 h were stained with TUNEL and PCNA.

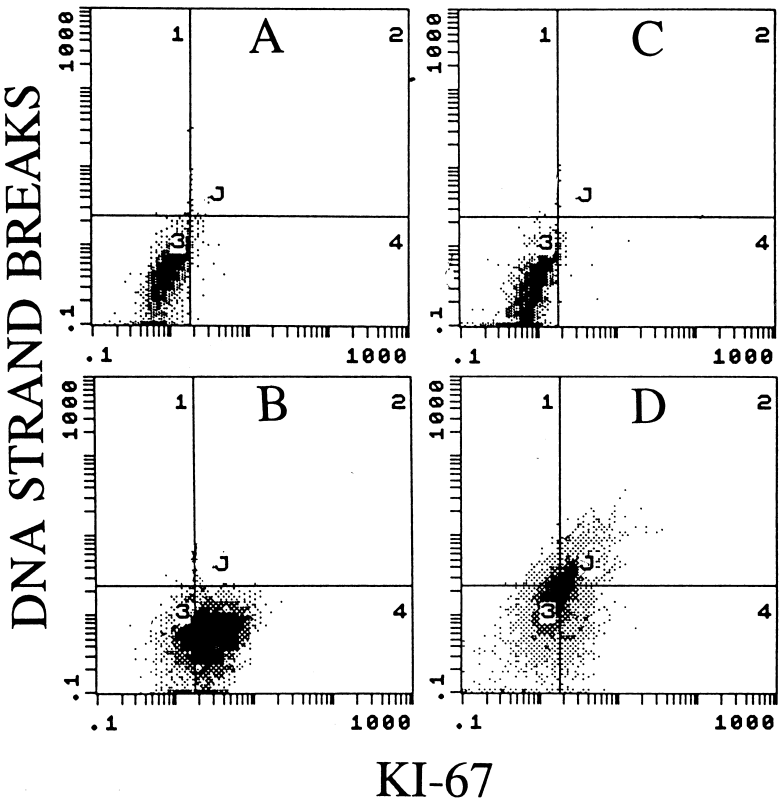

Fig. 2. DNA strand breaks and Ki-67 expression in HCT-15 colon carcinoma cells treated with sulindac sulfide. Cells were stained for Ki-67 and labeled with TUNEL, as in Section 2. Dot plots of TUNEL (ordinate) vs. Ki-67 (abscissa) are shown. Region 1, TUNEL(+)/Ki-67(−); Region 2, TUNEL(+)/Ki-67(+); Region 3, TUNEL(−)/Ki − 67(−); Region 4, TUNEL(−)/Ki-67(+). Panel A: the untreated cells were stained with TUNEL control solution and IgG_1 (Ki-67 isotypic control). Panel B: untreated cells were stained with TUNEL and Ki-67. Panel C: cells treated with SS 200 μM for 48 h were stained with TUNEL control solution and IgG1. Panel D: cells treated with SS 200 μM for 48 h were stained with TUNEL and Ki-67.

and the expression of proliferation markers (PCNA or Ki-67) can be assessed. The four regions, 1–4, were saved in listmode files. Region 3 was defined by the sample that represented the negative controls for both TUNEL and PCNA or Ki − 67 antigen (Fig. 1(A) and Fig. 2(A)).

Based on the expression of these antigens and the presence of DNA strand breaks, untreated HCT-15 and HT-29 cells could be separated into four subpopulations of cells, as described below [see also Figs. 1 and 2 (HCT-15); Table 2 (HCT-15) and Table 3 (HT-29)].

1. Cells that do not express proliferation markers and show DNA strand breaks [PCNA or Ki-67 (−)/TUNEL(+)]. These cells are 0.1–2% of the total (region 1, Fig. 1(B) and Fig. 2(B)). They are apoptotic cells.
2. Cells that express proliferation markers and show DNA strand breaks [PCNA or Ki-67 (+)/TUNEL(+). These cells are 1–1.7% of the total (region 2, Fig. 1(B) and Fig. 2(B)). The significance of this population is not conspicuously apparent.
3. Cells that do not express proliferation markers and have no detectable DNA strand breaks [PCNA or Ki-67(−)/TUNEL(−)]. They are 4–17% of the total (region 3, Fig. 1(B) and Fig. 2(B)). These are probably cells that have entered a stationary or quiescent phase, possibly following the logarithmic growth.
4. Cells that express proliferation markers and have no detectable DNA strand breaks [PCNA or Ki-67 (+)/TUNEL(−)]. These cells are ≥ 80% of the total (region 4, Fig. 1(B) and Fig. 2(B)). They

Table 2
DNA strand breaks and the expression of proliferation markers in HCT-15 colon cancer cells treated with sulindac sulfide (SS)

Time	24 h		48 h		72 h	
	PCNA(+)	PCNA(−)	PCNA(+)	PCNA(−)	PCNA(+)	PCNA(−)
TUNEL(+)						
Control	2%	0.2%	3%	0.3%	1.7%	0.4%
SS	12	7	28	7	38	4
TUNEL(−)						
Control	93	4	94	3	81	17
SS	43	28	42	23	32	26
	Ki-67(+)	Ki-67(−)	Ki-67(+)	Ki-67(−)	Ki-67(+)	Ki-67(−)
TUNEL(+)						
Control	3	2	3	0.2	1	0.7
SS	2.5	0.7	13	12	20	12
TUNEL(−)						
Control	91	3	94	3	83	15
SS	80	16	43	32	24	44

DNA strand breaks were determined by the TUNEL assay and the expression of the proliferation markers PCNA and Ki-67 were assayed by a triparameter flow cytometric analysis, as described in Section 2. This protocol was repeated three times and results were within 10% of the values presented here.

represent the rapidly proliferating fraction of these cells, which were in their growth phase.

We were able to determine the distribution of DNA content in the cell cycle phases only for the last population of cells, which provided the overwhelming majority of events on flow cytometry. For the remaining untreated cells, the events were too few to obtain an accurate DNA histogram. The role of populations 1 and 3 above was also deduced in conjunction with the changes that were observed after SS treatment (see below).

The expression of proliferation markers was time-

Table 3
DNA strand breaks and the expression of proliferation markers in HT-29 colon cancer cells treated with sulindac sulfide (SS)

Time	24 h		48 h		72 h	
	PCNA(+)	PCNA(−)	PCNA(+)	PCNA(−)	PCNA(+)	PCNA(−)
TUNEL(+)						
Control	2%	0.1%	2%	0.1%	1.7%	0.4%
SS	4.2	0.4	26	4	34	3.2
TUNEL(−)						
Control	90	7	90	7	80	17
SS	55	40	45	25	34	28
	Ki-67(+)	Ki-67(−)	Ki-67(+)	Ki-67(−)	Ki-67(+)	Ki-67(−)
TUNEL(+)						
Control	2	2	2	2	1	0.7
SS	2.5	0.7	29	8	37	6.8
TUNEL(−)						
Control	91	4	91	4	83	15
SS	79	18	17	46	20	35

DNA strand breaks were determined by the TUNEL assay and the expression of the proliferation markers PCNA and Ki-67 were assayed by a triparameter flow cytometric analysis, as described in Section 2. This protocol was repeated three times and results were within 10% of the values presented here.

dependent in untreated HCT-15 cells, as it decreased by about 10% between 48 h and 72 h. This decrease may reflect the fraction of cells that, during this time period, started completing their logarithmic phase of growth (Fig. 3, bottom panels and Table 2). Similar results were obtained for HT-29 cells, as indicated in Table 3.

3.3. The effect of sulindac sulfide on the four populations of colon cancer cells

Sulindac sulfide had a profound effect on the distribution of HCT-15 cells in the four different populations described above. Our findings, depicted in Figs. 1–3 and Tables 2 and 3, are summarized below. Treatment of HCT–15 cells with SS 200 μM led to the following changes:

1. Increase in the proportion of cells that do not express proliferation markers and show DNA strand breaks. At 72 h the PCNA(−)/TUNEL(+) cells increased to 4% of the total (control: 0.4%), whereas those that were Ki-67(−)/TUNEL(+) increased to 12% of the total (control: 0.7%).
2. Increase in the proportion of cells that express proliferation markers and show DNA strand breaks. At 72 h the PCNA(+)/TUNEL(+) cells increased to 38% of the total (control: 1.7%), whereas those that were Ki-67(+)/TUNEL(+) increased to 20% of the total (control: 1%).
3. Increase in the proportion of cells that do not express proliferation markers and have no detectable DNA strand breaks. At 72 h the PCNA(−)/TUNEL(−) cells increased to 26% of the total (control: 17%), whereas those that were Ki-67(−)/TUNEL(−) increased to 44% of the total (control: 15%). Of note, in this case the changes were more pronounced at 48 h.
4. Decrease in the proportion of cells that express proliferation markers and have no detectable DNA strand breaks. At 72 h the PCNA(+)/TUNEL(−) cells decreased to 32% of the total (control: 81%), whereas those that were Ki-67(+)/TUNEL(−) decreased to 24% of the total (control: 83%).

Overall, sulindac sulfide appears to have depleted the pool of proliferating cells and enhanced the other three populations of cells. This has been followed by a modest increase in the population of stationary cells (proliferation marker and TUNEL negative cells). However, the most significant shift of cells has taken place towards the TUNEL positive cells (proliferation marker positive and negative cells), which now comprise about half of all the cells.

HT-29 cells treated with SS 175 μM displayed similar changes as HCT-15 cells (Table 3).

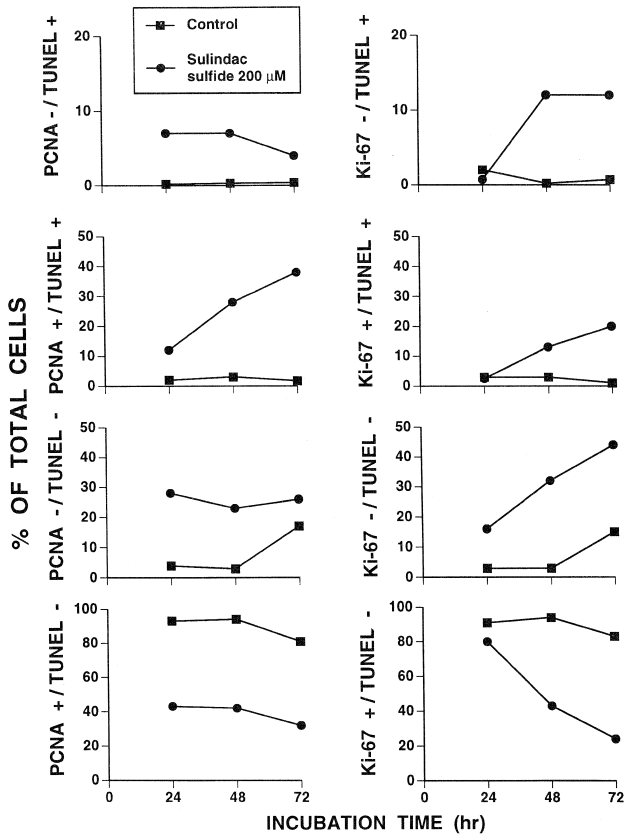

Fig. 3. The effect of sulindac sulfide on the subpopulations of HCT-15 cells defined by TUNEL and PCNA/Ki-67. This figure, based on the results shown in Tables 2 and 3, presents the percentage changes of the various cell subpopulations after treatment with SS for 24, 48, and 72 h. The definition of each population is described in Section 2.

3.4. Analysis of DNA content

To further characterize these populations of cells, we used gating analysis to determine their DNA distribution of HCT-15 cells. As demonstrated in Fig. 4, we were also able to study separately the two distinct subpopulations of region 4 of the two-dimensional histograms (e.g., Fig. 4, panel A).

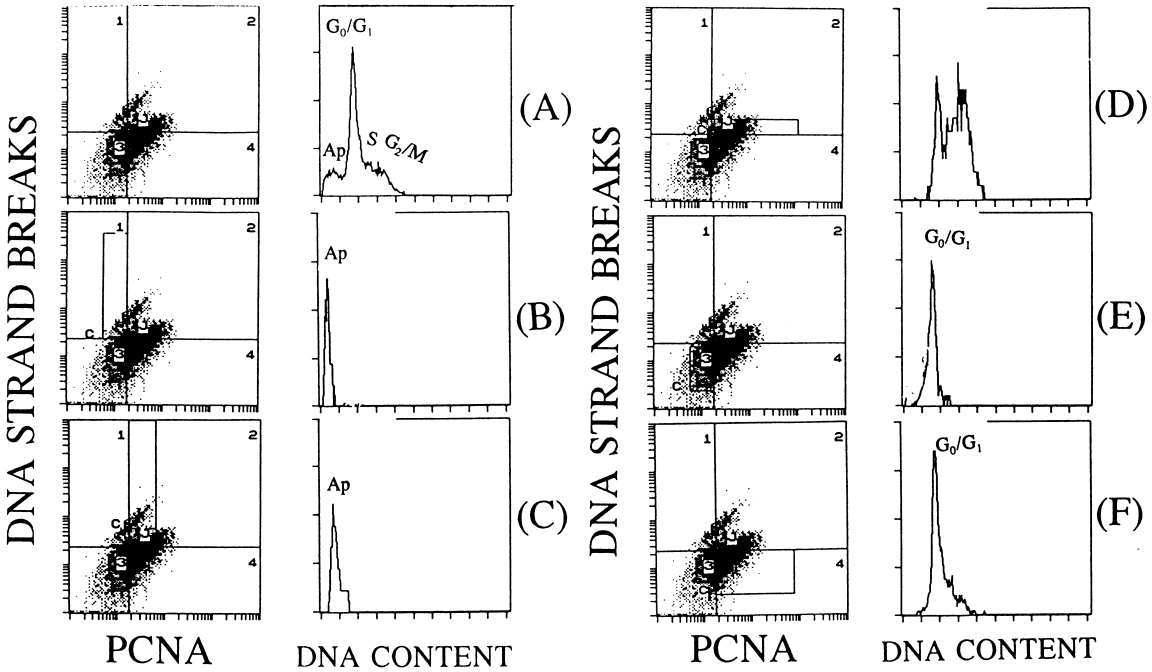

Fig. 4. Gating flow cytometric analysis of DNA strand breaks, PCNA expression and DNA content in HCT-15 cells treated with sulindac sulfide. HCT-15 cells were treated with SS 200 μM for 48 h and analyzed as in Section 2. Panel A shows the entire cell population distributed in four regions. The cells of gating region 1 [TUNEL(+)/PCNA(−)] displayed the subdiploid peak on DNA content analysis shown in panel B. The distinct cell population in the upper left portion of region 2 (Panel C, gated) which showed stronger TUNEL positivity, displayed a dominant subdiploid peak in the DNA histogram. The larger subpopulation of region 2 (right lower portion) displayed a DNA histogram with prominent G_1, S and G_2/M and no subdiploid peak (Panel D). Analysis of the TUNEL(−)/PCNA(−) region 3, showed a prominent G_0/G_1 phase (panel E). The cells in region 4 [TUNEL(−)/PCNA(+)] showed G_0/G_1 and S phases (panel F). The relative distribution of cells in the different phases of the cell cycle, measured by DNA content, is as follows: Panel A: Subdiploid (SD) = 13%; G_0/G_1 = 50%; S = 39%; G_2/M = 11%. Panel B: SD = 6%; G_0/G_1 = 0%; S = 0%; G_2/M = 0%. Panel C: SD = 7%; G_0/G_1 = 0%; S = 0%; G_2/M = 0%. Panel D: SD = 0%; G_0/G_1 = 20%; S = 55%; G_2/M = 25%. Panel E: SD = 0%; G_0/G_1 = 100%; S = 0%; G_2/M = 0%. Panel E: SD = 0%; G_0/G_1 = 70%; S = 30%; G_2/M = 0%.

In the case shown in Fig. 4, HCT-15 cells were treated with SS 200 μM for 48 h. Panel A shows that when the entire population of SS-treated cells were assessed for DNA content, there was (the expected) apoptotic peak. Panel B shows that cells that were TUNEL(+)/PCNA(−) manifested a dominant apoptotic peak, without discernible G_0/G_1, S and G_2/M phases. Cells that were TUNEL(+)/PCNA(+) consisted of two subpopulations: one subpopulation (7% of all the cells) contained cells that were more strongly TUNEL(+) and showed a dominant subdiploid apoptotic peak on DNA content analysis (panel C); the other subpopulation (21% of all the cells), which was less strongly TUNEL(+), consisted of cells with no subdiploid apoptotic peak and DNA content consistent with S and G_2/M phases of the cell cycle (panel D). Cells that were TUNEL(−)/PCNA(−) had a prominent G_0/G_1 peak and almost nonexistent S and G_2/M peaks (panel E). Finally, cells that were TUNEL(−)/PCNA(+) showed DNA content mainly in G_0/G_1 and S phases, with few cells in the G_2/M phase.

These findings indicate that the TUNEL(+)/PCNA(+) cell population, the one most prominently increased by SS, was heterogeneous. As evidenced by their DNA content, some of these cells were clearly apoptotic by DNA content criteria, whereas the rest displayed prominent S and G_2/M phases. The latter were about three-fold greater than the former. Similar results were obtained for HCT-15

cells at 72 h and also in the case of HT-29 cells (data not shown).

3.5. Sulindac sulfide induces similar changes in HL-60 promyelocytic leukemia cells

To ascertain whether these findings are restricted to colonic cells, we evaluated the effect of sulindac sulfide on the HL-60 promyelocytic leukemia cell line. As we have already shown [1], SS induces apoptosis in these cells as well. HL-60 cells were treated with SS 175 μM for 4 h, as previously described.

Table 4 shows the triparameter flow cytometric analysis of these cells for DNA strand breaks (TUNEL), PCNA or Ki-67 expression, and DNA content. Again, four populations of cells are identified, based on TUNEL positivity and PCNA/Ki-67 expression, in the same manner that was used in analyzing HT-29 and HCT-15 cells.

Similar to the findings in the two colon cell lines, SS significantly affects the distribution of HL-60 cells in these four populations. Particularly important is the increase in the cells that expressed proliferation markers and displayed DNA strand breaks, TUNEL(+)/Ki-67(+) or TUNEL(+)/PCNA(+), and the decrease in those cells that express proliferation markers but show no evidence of DNA strand breaks, TUNEL(−)/Ki-67(+) and TUNEL(−)/i PCNA(+). Also, the TUNEL(+)/PCNA(+) cells consisted of two subpopulations, one comprised of apoptotic cells and the other of cells with prominent S and G_2/M peaks.

Table 4
DNA strand breaks and the expression of proliferation markers in HL-60 promyelocytic leukemia cells treated with sulindac sulfide

	PCNA(+)	PCNA(−)
TUNEL(+)		
Control	5%	0.1%
Sulindac sulfide	24	8
TUNEL(−)		
Control	87	7
Sulindac sulfide	21	47
	Ki-67(+)	Ki-67(−)
TUNEL(+)		
Control	5	1
Sulindac sulfide	28	4
TUNEL(−)		
Control	72	22
Sulindac sulfide	42	26

The cells were treated with sulindac sulfide for 4 h. DNA strand breaks were determined by the TUNEL assay and the expression of the proliferation markers PCNA and Ki-67 were assayed by a triparameter flow cytometric analysis, as described in Section 2. This protocol was repeated twice and results were within 5% of the values presented here.

These findings clearly indicate that the type of response to sulindac sulfide obtained with the colon cells also takes place in the non-colonic cells such as leukemia cells.

4. Discussion

The findings of our present study provide insights into the effect of sulindac sulfide on colon cancer cells. First, they document the redistribution of cells among several different subpopulations, defined by the expression of proliferation markers and DNA strand breaks. And, second, they suggest that some clearly apoptotic cells can strongly express the proliferation markers PCNA and Ki-67.

Study of the two cell lines, HT-29 and HCT-15, with the triparameter flow cytometric analysis provided the simultaneous assessment of three important parameters, namely the expression of one proliferation marker, the extent of DNA strand breaks and also the DNA content of the cells. This approach permitted not only the unambiguous definition of several cell subpopulations but also the quantitative monitoring of the distribution of cells among them in response to SS treatment. This was not possible with assessment of these parameters separately as was done in our previous studies.

As one might expect, the overwhelming majority of control (i.e., untreated) cells in logarithmic growth were actively proliferating [PCNA/Ki-67(+)/TUNEL(−)], with few in quiescence [PCNA/Ki-67(−)/TUNEL(−)]; an insignificant fraction of cells (< 2%) were either apoptotic [PCNA/Ki-67(−)/TUNEL(+)] or had both DNA strand breaks and strong expression of proliferation markers [PCNA/Ki-67(+)/TUNEL(+)].

Sulindac sulfide profoundly altered the distribution of colon cancer cells among these four populations. First, it depleted significantly the proliferating fraction of cells [PCNA/Ki-67(+)/TUNEL(−)]. This

finding is in agreement with the antiproliferative effect of SS, which has been previously described [1] and was also documented in this study.

The second effect was the expected induction of apoptosis. Based on TUNEL positivity, over 30% of the cells are apoptotic. Further study of the TUNEL(+) cells on the basis of their DNA content, indicates that about 14% of the total at 48 h, had a subdiploid (apoptotic) peak. This number is consistent with previously published data [1,2]. That apoptotic cells express PCNA may be consistent with the reported role of PCNA in apoptosis. Sustained high levels of PCNA in conjunction with an arrest of the cell in G_1/S might be or might reflect one of the early signals for the cells to enter an apoptotic cycle [27].

The third effect was the increase in the population of quiescent cells. By 72 h, roughly one fourth [PCNA(−)/TUNEL(−)] to a little less than half [Ki-67(−)/TUNEL(−)] of the cells were quiescent. This represents a quantitatively major effect of SS on these cells, which contributes to its antiproliferative effect. Our previous analysis, based on determination of the DNA content of these cells and the expression of cell cycle regulatory proteins, detected but could not quantify this effect [1,28].

It appears that, as we described, sulindac sulfide reduces the final number of colon cancer cells by two effects: the induction of apoptosis and the induction of cell quiescence. Roughly, these two effects are quantitatively equivalent, with about one third of the cells becoming quiescent and another third becoming apoptotic. It is of interest that there was a marked difference in the effect of sulindac sulfide on the cell cycle distribution of the two cell lines we studied. While HT-29 cells arrested with diploid DNA content, HCT-15 cells retained their cell cycle distribution after treatment with this NSAID compound. It is conceivable that HCT-15 cells became ''quiescent,'' i.e., lost expression of proliferation markers, while maintaining their DNA content. The reasons for this difference remain unclear. Although both cell lines are derived from colon cancers, they differ, for example, in their genetic makeup, and in their ability to differentiate. HT-29 cells can differentiate in response to butyrate and glucose withdrawal, whereas HCT-15 cells show a limited response to such agents ([28,29] and unpublished observations).

Our results suggest that there may exist yet another category of cells, PCNA(+)/TUNEL(+). Of these, two subgroups can be distinguished, depending on the intensity of TUNEL and PCNA staining. The cells of the first group, representing about one-quarter of the total, exhibit strong TUNEL and weak PCNA staining and a prominent subdiploid peak (Fig. 4, Panel C). The cells of the second group (three quarters of the total) are weakly TUNEL-positive and strongly PCNA positive, have no appreciable subdiploid peak, and exhibit prominent S and G_2/M phase DNA contents. The cells in the first group are apoptotic where PCNA may be functioning in its role in DNA repair. The second group may represent cells in a very early stage of apoptosis when the DNA is beginning to be broken down by an endonucleolytic mechanism activated in the context of apoptosis. It is important to note that PCNA is associated with both DNA replication and DNA repair [23,30]. PCNA levels are elevated in response to DNA damage in vivo [31]; and the protein relocalizes to sites of DNA repair following damage in vitro [32,33]. However, Ki-67 may not be required for DNA excision repair, its expression being associated only with replicative DNA synthesis [31].

That these effects of sulindac sulfide were manifest in two different colon cancer cell lines and in one promyelocytic cell line, suggests that they may represent a more general activity of this versatile compound. Of further interest is that, in contrast to HT-29, the HCT-15 colon cancer cell line does not express either one of the cyclooxygenases (COX-1 or COX-2). Since these effects of SS are virtually indistinguishable in these two colon cell lines, it is unlikely that SS acts via its well recognized inhibition of prostaglandin synthesis. Alternative mechanisms must be sought, in accord similar notions concerning the NSAIDs [2].

The final important finding of our current work is that colon cancer and promyelocytic cells can be apoptotic and yet express the proliferation markers PCNA and Ki-67. Our triparameter flow cytometric analysis leaves no doubt that this is indeed the case; the apoptotic peak in DNA content histograms was the dominant component in cells expressing these markers. Though the reason for this is not entirely clear.

The presence of significant amounts of either Ki-67

or PCNA in apoptotic cells calls into question their specificity as proliferation markers. Our data indicate that their specificity may be limited in cases where apoptosis has been induced in cultured cells. However, it is consistent with the work of others that showed that proliferation markers, such as BrdU incorporation, can coexist in apoptotic cells [34]. Further work will be required to examine whether this is the case in tissues as well, or under the influence of additional apoptotic stimuli.

In conclusion, our data provide insight into the balance between the antiproliferative and apoptotic effect of the NSAID sulindac sulfide in cultured cells and indicate that apoptotic cells may strongly express the proliferation biomarkers Ki-67 and PCNA, thus suggesting that their specificity as proliferation markers may need to be re-assessed.

Acknowledgements

The authors wish to thank Allison M. Hawxhurst for editorial assistance. SJS is the Daniel Fraad Clinical Scholar. This project was supported by the American Cancer Society (#EDT-88 to BR) and the Arthur and Rochelle Belfer Foundation and The Rockefeller University Clinical Scholars fund (SJS).

References

[1] S.J. Shiff, L. Qiao, L.-L. Tsai, B. Rigas, J. Clin. Invest. 96 (1995) 491–503.
[2] R. Hanif, A. Pittas, Y. Feng, M.I. Koutsos, L. Staiano-Coico, S.J. Shiff, B. Rigas, Biochem. Pharmacol. 52 (1996) 237–245.
[3] P.J. Pasricha, A. Bedi, K. O'Connor, A. Rashid, A.J. Akhtar, M.L. Zahurak, S. Piantadosi, S.R. Hamilton, F.M. Giardiello, Gastroenterology 109 (1995) 994–998.
[4] G. Biasco, G.M. Paganelli, R. Santucci, G. Brandi, L. Barbara, J. Cell. Biochem. 19 (1994) 55–60.
[5] J. Gerdes, H. Lemke, H. Baisch, H.H. Wacker, U. Schwab, H. Stein, J. Immunol. 133 (1984) 1710–1715.
[6] D. McCormick, H. Chong, C. Hobbs, C. Datta, P.A. Hall, Histopathology 22 (1993) 355–360.
[7] J. Gerdes, Semin. Cancer Biol. 1 (1990) 199–206.
[8] R. Henriksen, P. Strang, T. Backstrom, E. Wilander, B. Tribukait, K. Oberg, Anticancer Res. 14 (1994) 603–608.
[9] T.P. Miller, T.M. Grogan, S. Dahlberg, C.M. Spier, R.M. Braziel, P.M. Banks, K. Foucar, C.R. Kjeldsberg, N. Levy, B.N. Nathwani, B. Schnitzer, R.R. Tubbs, E.R. Gaynor, R.I. Fisher, Blood 83 (1994) 1460–1466.
[10] G. Gasparini, P. Boracchi, P. Verderio, P. Bevilacqua, Int. J. Cancer 57 (1994) 822–829.
[11] F.J. Kubben, A. Peeters-Haesevoets, L.G. Engels, C.G. Baeten, B. Schutte, J.W. Arends, R.W. Stockbrugger, G.H. Blijham, Gut 35 (1994) 530–535.
[12] J.P. Neoptolemos, G.D. Oates, K.M. Newbold, A.M. Robson, C. McConkey, J. Powell, Brit. J. Surg. 82 (1995) 184–187.
[13] K.K. Shivji, M.K. Kenny, R.D. Wood, Cell 69 (1992) 367–374.
[14] L. Toschi, R. Bravo, J. Cell Biol. 107 (1988) 1623–1628.
[15] J.E. Celis, P. Madsen, FEBS Lett. 209 (1986) 277–283.
[16] H. Suzuki, K. Matsumoto, M. Terabe, J. Clin. Gastroenterol. 15 (1992) 317–320.
[17] G. Cattoretti, M.H. Becker, G. Key, M. Duchrow, C. Schluter, J. Galle, J. Gerdes, J. Pathol. 168 (1992) 357–363.
[18] M.P. Fairman, J. Cell Sci. 95 (1990) 1–4.
[19] W.E. Bolton, W.R. Mikulka, C.G. Healy, R.J. Schmittling, N.S. Kenyon, Cytometry 13 (1992) 117–126.
[20] J.F.R. Kerr, A.H. Wyllie, A.R. Currie, Brit. J. Cancer 26 (1972) 239–257.
[21] J.F.R. Kerr, B.V. Harmon, in: L.D. Tomei, F.O. Cope (Eds.), Apoptosis: The Molecular Biology of Cell Death, Cold Spring Harbor Laboratory Press, Plainview, NY, 1991, pp. 5–30
[22] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, J. Cell Biol. 119 (3) (1992) 493–501.
[23] P.J. Coates, V. Save, B. Ansari, P.A. Hall, J. Pathol. 176 (1995) 19–26.
[24] X. Li, F. Traganos, M.R. Melamed, Z. Darzynkiewicz, Cytometry 20 (1995) 172–180.
[25] R. Sgonc, G. Boeck, H. Dietrich, J. Gruber, H. Recheis, G. Wick, Trends Genet. 10 (1994) 41–42.
[26] P.S. Rabinovitch, R.M. Torres, D. Engel, J. Immunol. 136 (1986) 2769–2775.
[27] Y. Gazitt, G.W. Erdos, Cancer Res. 54 (1994) 950–956.
[28] Y. Goldberg, I.I. Nassif, A. Pittas, L-L. Tsai, B.D. Dynlacht, B. Rigas, S.J. Shiff, Oncogene 12 (1996) 893–901.
[29] A. Blais, Am. J. Physiol. 260 (1991) C1245–C1252.
[30] J. Wuarin, P. Nurse, Cell 85 (1996) 785–787.
[31] S. Pandey, E. Wang, J. Cell. Biochem. 58 (1995) 135–150.
[32] X. Li, M.R. Melamed, Z. Darzynkiewicz, Exp. Cell Res. 222 (1996) 28–37.
[33] P.A. Hall, P.H. McKee, H.D. Menage, R. Dover, D.P. Lane, Oncogene 8 (1993) 203–207.
[34] X. Wu, A.J. Levine, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 3602–3606.